Stroke

JOURNAL OF THE AMERICAN HEART ASSOCIATION

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STROKE/2006/472191 VERSION 1

This information is current as of September 12, 2006

Exhibit C

Author Disclosures

Yaniv Ziv: No disclosures

Arseny Finkelstein: No disclosures

Yona Geffen: No disclosures

Jonathan Kipnis: No disclosures

Igor Smirnov: No disclosures

Suzi Shpilman: No disclosures

Irena **Vertkin**: No disclosures

Michal Kimron: No disclosures

Aya Lange: No disclosures

Torsten Hecht: No disclosures

Klaus G Reyman: No disclosures

Jonathan B Marder: No disclosures

Michal Schwartz: No disclosures

Eti Yoles:
Institution/Employer: Proneuron Biotechnologies LTD., Amount: >= $10,000

A novel immune-based therapy for stroke induces neuroprotection and supports neurogenesis

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Acknowledgments

Y.G, I.S., S.S., I.V., M.K., A.L., J.B.M., and E.Y. are or were employees of Proneuron biotechnologies Ltd. The authors thank Dr. Elisheva Yonish-Rouach and Dr. Abraham Nyska for their constructive comments and fruitful discussions. M.S. is the incumbent of the Maurice and Ilse Katz Professorial Chair in Neuroimmunology. The work was supported by Proneuron Biotechnologies Ltd., Weizmann Science Park, Ness-Ziona, Israel.

Full title: A novel immune-based therapy for stroke induces neuroprotection and supports neurogenesis

Cover title: Immune-based therapy for stroke

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Keywords: stroke, neuroprotection, neurogenesis, reactive microglia

Background and purpose – The ability of the central nervous system (CNS) to cope with stressful conditions was shown to be dependent on proper T-cell mediated immune response. Because the therapeutic window for neuroprotection after acute insults such as stroke is relatively narrow, we searched for a procedure that would allow the relevant T cells to be recruited rapidly.

Methods – Permanent middle cerebral artery occlusion (MCAO) was induced in adult rats. Rats were treated with poly-YE using different regimens. Control and poly-YE-treated rats were assessed for functional recovery using neurological severity score (NSS) and Morris water Maze (MWM). Neuroprotection, neurogenesis, growth factor expression, and microglial phenotype were assessed using histological and immunofluorescence methods.

Results – Administration of poly-YE as late as 24 hours following MCAO yielded a beneficial effect manifested by better neurological performance, reduced neuronal loss, attenuation of behavioral deficits, and increased hippocampal and cortical neurogenesis. This compound affected the sub-acute phase by modulating microglial response, and increasing local production of insulin-like growth factor-I, known to be key player in neuronal survival and neurogenesis.

Conclusions – The relative wide therapeutic window, coupled with its efficacy in attenuating further degeneration and enhancing restoration, makes poly-YE a promising immune-based candidate for stroke therapy.

Introduction

Previously we have demonstrated that CNS trauma spontaneously evokes a beneficial, T cell-mediated immune response, which reduces neuronal loss[1]. In the damaged CNS tissue, CNS-specific T cells accumulate and become reactivated upon recognizing and interacting with their corresponding autoantigens presented to them by local antigen-presenting cells[2, 3]. This in turn leads to the production of specific neurotrophic factors (e.g. brain derived neurotrophic factor) by T cells, and to the proper activation of microglia/macrophages[4, 5]. Enhancing the activity of CNS-specific T cells (by a well-regulated passive or active immunization) was found to be beneficial in various animal models of CNS injuries[6-8]. The ability to maintain autoimmunity in healthy individuals, without developing autoimmune disease, was shown to be regulated by naturally occurring $CD4^+CD25^+$ regulatory T cells. However, recovery from CNS injury can benefit from a transient elimination or decreased activity of these cells[9]. Compounds that can down-regulate the constitutive suppression of regulatory T cells (Treg) were therefore, considered by us as potential candidates.

We found that poly-YE, a high-molecular-weight (22–45 kD) copolymer that was shown to exert modulatory effects on the immune system[10, 11], is capable of down-regulating the activity of the regulatory T cells, and utilized this copolymer to facilitate the spontaneous response of effector T cells recognizing antigens associated with a CNS insult. Our findings showed that a single administration of poly-YE in rats subjected to an ischemic stroke (permanent middle cerebral artery occlusion (MCAO)) produced long-lasting clinical and behavioral benefits, along with neuroprotection and increased neurogenesis, starting from the subacute phase.

Materials and Methods

Animals. Sprague-Dawley (SPD) male rats, aged 8–12 weeks, and C57Bl/6 male mice, aged 7-8 weeks, were supplied by Harlan Biotech (Jerusalem, Israel) and were handled according to the ARVO resolution on the use of animals in research. The study was approved by the IAUC of Proneuron Biotechnologies, Ltd., and the National Ethics Committee of the Israeli Council on Animal Experimentation.

Permanent middle cerebral artery occlusion in rats. Middle cerebral artery occlusion was preformed using a nylon filament as previously described[12].

Measurement of lesion size. The effect of poly-YE on early infarct volume was measured 48 h after MCAO and treatment with 0.5 mg poly-YE/rat or vehicle. From each brain, six 2-mm-thick coronal slices were cut with the aid of a brain slicer matrix (Braintree Scientific, Braintree, MA) and stained with 2% triphenyl tetrazolium chloride, as described elsewhere [13]. For testing the effect of poly-YE on cell loss at late stages (six weeks after MACO), brains were sectioned while frozen at 50 μm and stained with cresyl violet. The cross sectional area of both the intact and stroked hemisphere was measured using unbiased stereology procedure. The area of the infarcted hemisphere was subtracted from the area of the intact hemisphere and expressed as a percentage of the intact hemisphere. This was done in 13 evenly spaced sections through the brain and an average percent of tissue loss was analyzed.

Treatment with poly-YE. Upon completion of surgery the rats were randomly assigned to different treatment groups. All rats were injected once subcutaneously (s.c.) at the indicated times either with the designated dosages of poly-YE (Sigma-Aldrich), St. Louis, MO) in a final volume of 1 ml PBS or with PBS only (control). Rats were weighed before MCAO and twice weekly thereafter over the indicated time period.

Neurological assessment. Neurological severity scores (NSS) were computed as the sum of a number of ratings assigned to each of 11 parameters of posture and locomotion [14]. The rats were evaluated twice a week, from 24 h after MCAO until the end of the experimental period, by an investigator who was blinded to their treatment.

Testing of behavior in a Morris water maze (MWM). Starting 21–23 d after occlusion, rats were given three trials per day, on 4 consecutive days, to find a hidden platform located 1.5 cm below the water surface in a pool 1.4 m in diameter, as previously described[15]. On day 5 the platform was placed above the water level, where it was visible, and each rat was allowed up to 120 s to find and climb onto it. In all trials, timed movements of the rats were recorded using an EthoVision automated tracing system (Noldus Information Technology, Wageningen, The Netherlands).

Administration of 5-bromo-2'-deoxyuridine and tissue preperation. BrdU was dissolved in PBS (6.25 mg/ml) and injected i.p. into rats (50 mg/kg body weight) twice daily from day 7 to day 9 after MCAO. On day 28 the rats were deeply anesthetized and killed by transcardial perfusion with PBS and then with 4% paraformaldehyde.

Immunohistochemistry. For BrdU staining, tissue sections (25-μm width) were washed with PBS and incubated for 30 min in 2N HCl at 37°C. Sections were blocked for 1 h with blocking solution (PBS containing 20% normal horse serum and 0.5% Triton X-100). Tissue sections were then stained overnight with the primary antibodies (Abs): rat anti-BrdU (1:100; Oxford Biotechnology, Kidlington, Oxfordshire, UK), rabbit anti-MAP-2 (1:1000; Chemicon, Temecula, CA), goat anti-doublecortin (anti-DCX; 1:200; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-β-III tubulin (1:1000, Chemicon), and mouse anti-neuron-specific nuclear protein (anti-NeuN; 1:150; Chemicon). For labeling of microglia we used FITC-conjugated *Bandeiraea simplicifolia* isolectin B4 (IB-4, 1:50; Sigma-Aldrich) and mouse anti-class II major histocompatibility complex protein (anti-MHC-II; 1:40) Abs

(IQ Products, Groningen, The Netherlands). For labeling of astrocytes/progenitor cells we used mouse anti-Nestin (1:1000; Chemicon) and rabbit anti-glial fibrillary acidic protein (anti-GFAP) Abs (1:200; DAKO, Glostrup, Denmark). For detection of IGF-I expression we used goat anti-IGF-I Ab (1:20; R&D Systems, Minneapolis, MN). Secondary antibodies were Cy-2-conjugated donkey anti-mouse, Cy-3-conjugated donkey anti-mouse, Cy-3- or Cy-5-conjugated donkey anti-rat, and Cy-3-conjugated donkey anti rabbit (1:200; Jackson ImmunoResearch, West Grove, PA).

Quantification. Loss of hippocampal neurons was measured in brains that were removed from euthanized rats, promptly immersed in 10% buffered formalin for 24 h (to avoid autolysis), and then transferred to 70% alcohol. Coronal hippocampal slices (100 μm thick) were cut with the rat brain matrix and were stained with hematoxylin and eosin. All stained necrotic cells in the CA1, CA2, and CA3 hippocampal regions of the ischemic and contralateral sides of each slice were counted under a light microscope, and the number of necrotic cells per slice was calculated for each hemisphere. Data were averaged over 2–4 hippocampal slices for each rat. For immunohistochemical analysis of cell proliferation and hippocamppal neurogenesis, six coronal sections per rat brain, spanning the entire dentate gyrus were taken. Proliferation and differentiation were assessed using confocal microscopy (Zeiss LSM 510) by counting cells that were double-labeled with BrdU and NeuN in the dentate gyrus. An estimate of the total number of labeled cells per dentate gyrus was obtained as previously described[15]. Cortical neurogenesis was evaluated by counting cells that were double-labeled with BrdU and MAP-2, DCX, or Nestin in the ipsilateral cortex. At least two coronal sections per rat were included in the analysis of cortical neurogenesis. To quantify cortical neurogenesis, the total combined volume of the selected sections was multiplied by the number of cells counted per unit surface area of each histological section. IGF-I immunoreactivity was assayed by measurement of the fluorescence intensity per unit area

using ImageProPlus software. The same software was used to measure the areas occupied by MHC-II-stained and IB-4-stained cells.

Statistical analysis. Statistical calculations were performed using standard functions of Microsoft Excel, supplemented with the StatistiXL add-in package, which provides functions for advanced General Linear Model and nonparametric analyses. Because of large differences in variance between groups, and to obviate the need for data transformation, the data presented in Figure 3 were analyzed using non-parametric statistics. The data presented in Figure 4 were transformed to a normal distribution by application of an empirically optimized function ($\sqrt[3]{Y-1800}$), and the data for the ipsilateral and contralateral dentate gyrus of each rat were separately assessed by repeated-measures ANOVA.

Results

Poly-YE improves the neurological outcome after stroke

Using an in vitro bioassay for assessment of Treg activity[16, 17], we tested the possible effect of poly-YE on effector T cells (Teff) and Treg cells. We found that poly-YE down-regulates the suppressive effect of naturally occurring regulatory T-cells, and changes the cytokine-secretion profile of the activated Treg cells (see *Supplementary Figure 1 online*). We tested the therapeutic effect of s.c.-injected poly-YE in a rat model of permanent MCAO by examining neurological functions (measured by the NSS) over a period of 2 weeks. In three separate experiments poly-YE (0.5 mg per rat) or PBS (control) was injected immediately after MCAO (Figure 1A), or with a delay of 6 h (Figure 1B) or of 24 h (Figure 1C). All rats (including controls) showed some recovery with time, reflected by a substantial reduction in NSS. Neurological recovery in all three experiments was significantly better in the poly-YE-treated rats than in the controls (Repeated measures ANOVA; $p = 0.0013$, $p =$

0.0136, and $p = 0.0451$ for injections given immediately, 6 h after MCAO, and 24 h after MCAO, respectively). Repeated measures ANOVA disclosed a highly significant treatment effect ($p = 7 \times 10^{-6}$), while indicating that the time of injection was not a significant factor ($p = 0.87$ for poly-YE, $p = 0.44$ for PBS, $p = 0.81$ for pooled data). Thus, poly-YE injection significantly improved neurological recovery even when delayed for as long as 24 h after MCAO.

Next we injected rats, 24 h after MCAO, with different doses of poly-YE or PBS and assessed the NSS over a period of 6 weeks to determine whether the effect of the treatment is long lasting (Figure 1D). A significant dose-response treatment effect was evident (P=0.044, repeated measures ANOVA), with poly-YE at doses of 0.5mg/rat and 1mg/rat resulting in significantly lower NSS scores at the end of the experiment (Student-Neuhls-Krahl tests performed for $p = 0.05$). After 6 weeks the residual impairment in the treated rats was only half of that in the controls, implying that poly-YE treatment had long lasting effects. Body weights of the rats were monitored for 6 weeks after MCAO as an indicator of their general health. Rats treated with poly-YE showed, on the average, a smaller initial weight loss and a larger overall weight gain (Figure 1E, $p = 0.041$, ANOVA). Poly-YE effect on body weight gain was associated with neurological improvement in the two higher dosages (1 mg/rat and 0.5 mg/rat) while the lower dosage (0.15 mg/rat) seemed to be ineffective.

Poly-YE attenuates behavioral deficits after stroke

In addition to neurological activity, we assessed the long-term effect of a single injection of poly-YE on behavioral and cognitive functions using the Morris water maze (MWM). Rats were injected with poly-YE or PBS immediately after MCAO, and 3 to 3.5 weeks later they were trained for 4 d to locate and mount a hidden platform in the water maze. During the training period the MCA-occluded rats showed significant deficits in spatial learning abilities relative to naïve rats, often failing to acquire the ability to complete the task. No significant

effect of poly-YE treatment was detectable at this stage (results not shown). Sham-operated rats completed the task normally, indicating that the poor performance of the MCA-occluded rats was related to the damage induced by MCAO rather than general postoperative trauma. On day 5 the platform was raised so that it was visible above the water surface, and each MCA-occluded rat was allowed one attempt to locate and mount it. Rats injected with PBS (control) performed poorly, with 7 of the 12 rats swimming randomly around the platform and failing to complete the task within the allotted time (Figure 2A). Data were analyzed in terms of reciprocal time, corresponding to 'rate' of task completion. Rats treated with poly-YE performed significantly better (p = 0.041, two-tailed t-test, Figure 2B). There were no differences in swimming velocity between the groups (p = 0.19, two-tailed t-test; 25.4 ± 2.2 $cm.s^{-1}$ in the control group and 19.6 ± 2.9 $cm.s^{-1}$ in the poly-YE-treated group), indicating that the difference was behavioral rather than physical[18]. This impression was reinforced by the strong negative correlation between swimming velocity and reciprocal time (Pearson's correlation; r = - 0.85), with the fastest swimming observed in the rats that took the longest to complete the task.

Poly-YE attenuates hippocampal cell loss

We speculated that treatment with poly-YE would mainly target degenerative processes that occur after the hyper-acute phase, and thus we were not expecting to detect a short-term effect on infarct volume. Infarct volumes were measured 48 h following MCAO and immediate treatment (PBS, n = 11; 1 mg poly-YE, n = 14), using the triphenyl tetrazolium chloride histological staining technique. Differences in infarct volume between controls and treated rats were not significant (mean ischemic volumes ± SD were 178 ± 110 mm^3 and 200 ± 104 mm^3, respectively; p = 0.614, two-tailed t-test). Nevertheless, the extent of tissue loss measured six weeks after MCAO tended to be greater in the control rats (n =

10) than in poly-YE treated rats (n = 11) (mean values of lost volume ± SD were 40 ± 21 % and 25 ±14 %, respectively; $p = 0.064$, two-tailed *t*-test).

Brain damage after ischemia results from pathophysiological events in the infarct area and the penumbra, and in remote brain regions, such as the hippocampus[19]. Rats were subjected to MCAO followed immediately by injection of PBS or 1 mg of poly-YE. Histological examination of the hippocampus in the damaged (ipsilateral) side of the brain 48 h later showed large numbers of necrotic cells in the PBS-treated controls, whereas in the poly-YE-treated rats their numbers were significantly reduced (Figure 3A,B). The reduction in cell necrosis induced by poly-YE treatment was so dramatic that the ipsilateral and contralateral sides were indistinguishable ($p = 0.25$, Wilcoxon two-tailed test).

Poly-YE promotes neurogenesis after stroke

Recent studies have shown that immune cells play a key role in maintaining adult neurogenesis, and that T cell-activated microglia can induce neuronal differentiation from adult neural progenitor cells[15, 20]. We therefore examined whether treatment with poly-YE, in addition to its neuroprotective action, could also induce neurogenesis.

In rats subjected to MCAO and then immediately treated with PBS or 1 mg of poly-YE, neurogenesis in the hippocampus was assessed by injecting the cell proliferation marker BrdU. Because BrdU is known to exert cytotoxic effects on proliferating lymphocytes[21], it was administered only on days 7 through 9 after MCAO. Rats were killed 28 d after MCAO, and neurogenesis was evaluated by staining of coronal hippocampal sections with antibodies against BrdU and NeuN (a marker of post-mitotic neurons). We counted the newly formed $BrdU^+/NeuN^+$ cells in the granular cell layer of the hippocampal dentate gyrus of poly-YE-treated and control rats (Figure 4). An overall increase in the number of newly formed neurons was found in the dentate gyri of poly-YE treated rats relative to PBS treated controls (Figure 5A, $p = 0.047$, repeated measures ANOVA). Notably, the increase was observed in

both ipsilateral and contralateral sides, suggesting that a systemic response (e.g. via CNS-specific T-cells), facilitated by poly-YE, supports neurogenesis in a site not directly affected by MCAO. Figure 4B,C shows representative confocal micrographs of $BrdU^+/NeuN^+$ cells in the dentate gyrus.

Studies have demonstrated that ischemic or traumatic brain injury can induce neurogenesis in regions of the brain that are normally non-neurogenic [22]. We stained coronal sections containing the cortical lesion site for BrdU and the progenitor-cell marker Nestin. Numerous Nestin-stained cells could be seen surrounding the lesion site 28 d after MCAO (Figure 5A), most of them co-labeled with BrdU (Figure 5B) and the astrocytic marker glial fibrillary acidic protein (GFAP; not shown). Within the lesion site, no viable neurons (expressing the neuronal marker MAP-2) were detectable (Figure 5A). Because opinions are divided as to whether cortical neurogenesis is feasible[22], we included several neuronal markers in our analysis and used 3-D confocal scanning microscopy to examine their co-localization with BrdU. We detected newly formed cells co-labeled with BrdU and the early neuronal differentiation markers β-III tubulin and doublecortin (DCX) (Figure 6C, D). We could also detect newly formed neurons with mature phenotypes, indicated by BrdU/NeuN (Figure 5E) and BrdU/MAP-2 double staining (Figure 5F-I). Some of the $BrdU^+/MAP\text{-}2^+$ neurons were seen within the area adjacent to the lesion (Figure 5F) and had short processes (Figure 5G), implying that conditions in that area were not optimal for cell growth and connectivity. In contrast, newly formed mature neurons with highly developed pyramidal morphology were detected in cortical areas more remote from the lesion (Figure 5H,I). The dense and uniform BrdU labeling, found on neurons in both early and late maturation stages, suggests that it did not result from apoptosis or DNA repair.

To avoid possible artifacts caused by nonspecific fluorescence staining of myelin debris which might interfere with discrete staining of neuronal nuclear markers, we chose to

quantify $BrdU^+$ cells co-labeled with MAP-2 or DCX, which have a clear dendritic morphology. We took special care to count only those cells that exhibit the typical neuronal morphology and in which BrdU staining appeared in the center of the cell. Quantitative analysis revealed an increase of almost threefold in the numbers of newly formed neurons with a mature phenotype ($BrdU^+/MAP\text{-}2^+$) in the cortices of poly-YE treated rats relative to PBS-treated rats (Figure 5J). However, the numbers of newly differentiating neurons ($BrdU^+/DCX^+$) and progenitor cells/proliferating astrocytes ($BrdU^+/Nestin^+$) were not significantly different between the poly-YE treated rats and the controls (Figure 5 J).

In addition to its effect on neurogenesis, we found that poly-YE induced long-lasting changes in microglial phenotype in the thalamus, which were associated with increased IGF-I expression by $Nestin^+/GFAP^+$ cells (see *Supplementary Figure 1 online*).

Discussion

The results presented above show that administration of poly-YE as late as 24 h after the induction of ischemic stroke greatly improves subsequent recovery. The therapeutic effect of poly-YE was demonstrated in rats by neurological criteria (NSS scores), behavioral parameters (MWM task), and weight gain. The beneficial effects of poly-YE include both prevention of ischemia-induced remote, delayed degeneration (neuroprotection), and enhancement of repair (neurogenesis). The therapeutic time window (of 24 h) seemed to be significantly wider than that of most of the current and candidate therapies for stroke.

Post injury immune response has often been perceived as part of the detrimental cascade that causes neuronal death and impairs neurogenesis[23-25]. Consequently, a number of anti-inflammatory agents have been tested in animal models of stroke, with the goal of limiting the recruitment and activation of inflammatory cells[24]. This approach was indeed found to

reduce infarct size and neurological deficits if applied within a few hours of ischemic onset. However, a controlled clinical trial with the anti-inflammatory corticosteroid methylprednisolone resulted in an unexpected increase in short-term mortality[26].

We have proposed that resident microglia and circulating CNS-specific T cells contributes to CNS plasticity[1, 15, 27]. Importantly, the activity of such immune cells can be detrimental if not well regulated[28, 29]. In line with this concept, a study in humans demonstrated an association between the incidence of myelin-specific T cells and improved recovery from traumatic brain injury[30].

Recent studies have shown that ischemic brain injury can trigger neural cell renewal also in areas outside of the 'classical' neurogenic compartments (the hippocampal dentate gyrus and the subventricular zone)[22], including in the post-stroke human brain[31]. Indeed, the neurogenesis-promoting effect of poly-YE treatment was evident in both the hippocampus and in the cerebral cortex. The overall increase in cortical neurogenesis after poly-YE treatment might account for part of the observed behavioral and neurological improvement. The fact that increased number of new neurons with mature phenotype was detected in the hippocampus and cortex of the poly-YE-treated almost 3 weeks after BrdU injection suggests that the effect of poly-YE on neurogenesis is long lasting rather than transient.

The observed increase in IGF-I production by Nestin$^+$ cells and the higher incidence of MHC-II$^+$ microglia/macrophages seen in the thalamus of the poly-YE-treated rats further suggest that immunomodulation, which allows a well-controlled T cell-mediated response, contribute for creating neurogenic conditions after an ischemic brain insult.

At this stage, we cannot rule out the possibility that immune-cell populations other than Treg cells are also affected by poly-YE in vivo, as other studies have shown that this compound can induce activation of γδ-T cells in vitro[10]. Regardless of the specific T-cell subtypes directly affected by poly-YE, further studies are needed to define where the

dialogue between poly-YE-induced immune response and the injured CNS takes place (i.e. peripheral lymph nodes, cerebrospinal fluid, injured parenchyma).

In conclusion, the findings of this study introduce poly-YE as a novel immune-based therapy for the treatment of complex acute neurodegenerative disorders such as stroke. Our findings emphasize the feasibility of therapeutic intervention at the sub-acute phase if a proper choice of the therapy is made. Given its immunomodulatory mode of action and its remarkably wide therapeutic window, poly-YE can probably be used concomitantly or sequentially with other therapies for stroke.

Figure legends

Figure 1. Administration of poly-YE at different times after MCAO. (**A**) Immediately, (**B**) 6 h after MCAO, and (**C**) 24 h after MCAO, each rat was injected s.c. with either PBS (control) or 0.5 mg of poly-YE. Data points represent neurological severity scores (NSS) (mean ± SEM) from day 1 to day 14 after MCAO. (**D**) Rats were injected, 24 h after MCAO, with PBS or with 0.15, 0.5, or 1 mg of poly-YE. Shown are NSS values (means ± SEM) recorded before treatment (day 1), 1 d after treatment (day 2), and up to 6 weeks after treatment. (**E**) Gain in body mass values (means ± SEM) from the day of MCAO in rats treated with PBS or with 0.15, 0.5, or 1 mg of poly-YE/rat.

Figure 2. Performance of ischemic rats in the Morris Water Maze visible platform task. (**A**) Examples of swimming paths of two PBS-injected ischemic rats (control) that failed to complete the task in 120 s and two ischemic rats treated with 0.5 mg poly-YE that completed the task within this time frame. The small central disk in each diagram represents the location of the visible platform, and lines trace the rats' swimming paths. (**B**) Data analyzed as

reciprocal time, corresponding to 'rate' of task completion, with rats that failed to complete the task in 120 s given a rate score of 0 s^{-1} (* $p = 0.041$, two-tailed t-test).

Figure 3. Effect of poly-YE on hippocampal cell death after stroke. (**A**) Total numbers of necrotic cells per section in the CA1, CA2, and CA3 hippocampal regions (*$p = 0.016$, Mann-Whitney two-tailed test; $n = 5$ and $n = 4$ for PBS-treated and poly-YE-treated rats, respectively). Data are means ± SEM. (**B**) Densely stained necrotic cells are arrayed around the hippocampus on the ischemic side in a coronal section from the hippocampus of a control rat (left) and a poly-YE-treated rat (right), 48 h after MCAO (bar = 500 μm).

Figure 4. Increased hippocampal neurogenesis in poly-YE-treated rats. Starting on day 7 after MCAO, poly-YE-treated and PBS-treated rats were injected twice daily for 3 days with BrdU. On day 28 the rats were killed and their brains were analyzed for hippocampal neurogenesis. (**A**) Quantification of $BrdU^+/NeuN^+$ double-labeled cells in the dentate gyrus 28 d after MCAO ($n = 6$). Data are means ± SEM. (B) Representative confocal micrographs of the dentate gyrus of poly-YE-treated and PBS-treated rats double-stained for BrdU (green) and NeuN (red) (bar = 100 μm) (**C**) Higher magnification images of two 3-week-old neurons in the granular cell layer of the dentate gyrus of a poly-YE-treated rat showing BrdU (green) and NeuN (red) staining, separately or as a merged image (bar = 20 μm).

Figure 5. Poly-YE enhances stroke-induced cortical neurogenesis (**A**) Sections containing cortical structures affected by the stroke were stained for BrdU (red), Nestin (blue), and MAP-2 (green) (bar = 200 μm). $Nestin^+$ cells delineate the cortical lesion (schematically depicted in pink) and separate it from less affected areas, in which most cortical neurons were $MAP\text{-}2^+$. (**B**) The majority of $Nestin^+$ cells demarcating the cortical lesion were also co-

labeled with BrdU (bar = 50μm). (**C**) Orthogonal confocal images of new immature neurons found in proximity to the lesion site, stained for BrdU/□-III tubulin, and (**D**) for BrdU/DCX. (**E**) Cells with typical neuronal morphology that were labeled with BrdU and expressed NeuN or MAP-2 were defined as mature newly formed neurons. (**F**) Some of the $BrdU^+/MAP\text{-}2^+$ neurons had short processes and appeared mainly adjacent to the lesion area (bar = 50 μm). (**G**) Higher magnification of the area marked in (**F**). (**H**) $BrdU^+/MAP\text{-}2^+$ cells with long processes and a pyramidal morphology appeared at a distance of ~150-400 μm from the lesion. (**I**) These newly formed neurons were often seen as an integral part of the local neuronal network, as demonstrated by representative sequential confocal images of a $BrdU^+/MAP\text{-}2^+$ cell along the 'z' plane. (**J**), Treatment with poly-YE enhances cortical neurogenesis. For quantitative analysis, sections were stained for BrdU, Nestin, DCX and MAP-2. 3-D confocal scanning microscopy was used to verify co-localization of each $BrdU^+/MAP\text{-}2^+$, $BrdU^+/DCX^+$, and $BrdU^+/Nestin^+$ cell in the vicinity of the cortical lesion ($^*p < 0.05$, *t*-test; $n = 4$). Data are means ± SEM. Scale bars are 20 μm, unless stated otherwise.

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Figure 1

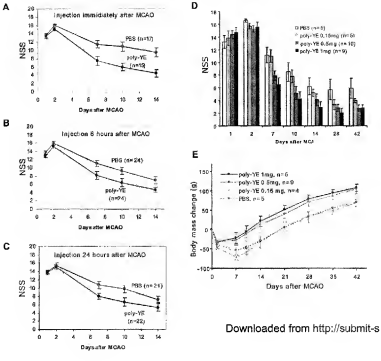

Figure 2

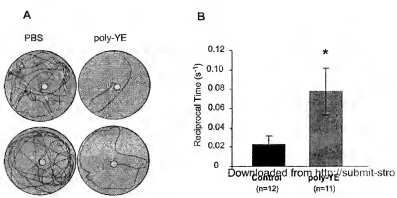

Figure 3

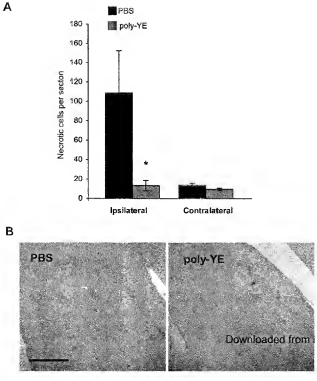
A
PBS
poly-YE
Necrotic cells per section
180
160
140
120
100
80
60
40
20
0
*
Ipsilateral
Contralateral
B
PBS
poly-YE
Downloaded from

Figure 4

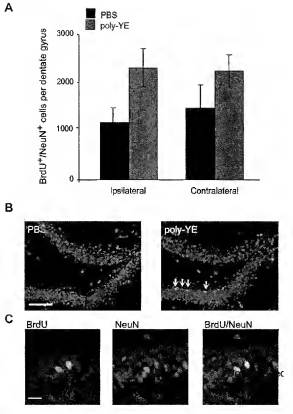
A
PBS
poly-YE
BrdU+/NeuN+ cells per dentate gyrus
3000
2000
1000
0
Ipsilateral
Contralateral
B
PBS
poly-YE
C
BrdU
NeuN
BrdU/NeuN

Figure 5